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APPLICATION NUMBER: 60/139,788

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## PRIORITY DOCUMENT

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## PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2)

Docket Number		11004-1		Type a Plus Sign (+) inside this Box -->		+	
INVENTOR(S)/APPLICANT(S)							
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TITLE OF THE INVENTION (280 CHARACTERS MAX.)							
Oil Content And Composition enhancement by Targeted/Non-Targeted Expression Of Plant Plastidial And Bacterial Gene For Glycerol-3-Phosphate Acyltransferase							
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STATE		Ontario		ZIP CODE		K1A 0R6 COUNTRY Canada	
ENCLOSED APPLICATION PARTS (Check all that Apply)							
<input checked="" type="checkbox"/> Specification		Number of pages		10		<input type="checkbox"/> Small Entity Statement	
<input checked="" type="checkbox"/> Drawing(s)		Number of Sheets		8		<input type="checkbox"/> Other (specify)	
METHOD OF PAYMENT (Check One)							
<input type="checkbox"/> A check or money order is enclosed to cover the Provisional filing fees						Provisional Filing Fee Amount (\$)	
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 14-0429						\$ 150.00	

The invention was made by an agency of the United States Government or under contract with an agency of the United States Government. ☒ No

Date: 21 June 1999.

Respectfully submitted,

J. Wayne Anderson  
Patent Agent for Applicant  
Regn No. 28,158Enclosures  
:ech\_\_\_\_ Additional inventors are being named a separately numbered sheets attached hereto.  
PROVISIONAL APPLICATION FILING ONLY

Jc541 U.S. PTO

60/139788

06/21/99

OIL CONTENT AND COMPOSITION ENHANCEMENT BY  
TARGETED/NON-TARGETED EXPRESSION OF PLANT PLASTIDIAL AND  
BACTERIAL GENE FOR GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE

5

There is a general industrial demand for higher oil content in living organisms in order to be more cost effective during production and processing. It is estimated that a 1% increase in seed oil would add \$20 M to the value of the Canadian canola crop at the farm gate.

10

One way of accomplishing it is by modifying metabolic pathways of living organisms by enhancing the expression of genes involved in oil synthesis. For example, overexpression of a yeast SLC1-1 has been found to increase oil content in plants (Zou et al.,<sup>1</sup> 1997).

15

Only a limited number of genes have been reported to enhance oil content (for example, Zou et al.<sup>1</sup>, 1997, Marillia et al.<sup>2</sup>, 1998). There is a need to explore other approaches using other genes which might be more effective alone or in combination with previously cloned genes.

20

We propose to increase oil content and modify oil composition by overexpressing the genes for the enzyme glycerol-3-phosphate acyltransferase (GPAT). This has been achieved in yeast. Details of the procedures and findings are provided below.

25

This is the first demonstration of an increase in carbon flux into triglycerides by manipulating the expression of a GPAT enzyme.

30

The invention is immediately applicable to the improvement of processes in which commercially important fatty acids (e.g. gamma linolenic acid) and cocoa butter are produced in yeast.

## BACKGROUND

- GPAT is the first enzyme of membrane and storage lipid (triacylglycerol; TAG) biosynthesis in living organisms. It determines the type of fatty acid incorporated in the *sn*-1 position of membrane and storage lipids. Overall, its specificity for various acyl-CoA appears to be flexible (Frentzen, <sup>3</sup> 1993; Frentzen and Wolter, <sup>4</sup> 1998). Since it is the first step in triglyceride and phospholipid lipid biosynthesis, it is surmised that enhancing production of GPAT might also result in an increase of 1-acylglycerol-3-phosphate through the Kennedy pathway, resulting in higher oil content. Three types of GPAT have been reported from plants: plastidial (P), mitochondrial (M), and cytosolic (ER). They exhibit variable specificity toward acyl-CoA and acyl-ACP substrates (Frentzen, <sup>3</sup> 1993). The ER form of GPAT is considered to be the most important for TAG manipulation but it has not been possible to isolate a gene for this enzyme. However, cDNAs for P-GPATs have been isolated and characterized from a number of species. Also, heterologous GPAT genes from bacteria and mammals are available (see Wilkison and Bell <sup>5</sup>, 1997; Dircks and Sul, <sup>6</sup> 1997).
- A plastidial GPAT gene has been used by others to change the fatty acid composition of membrane lipids and to improve chilling tolerance (Murata, et al, <sup>7</sup> 1992 and Nishizawa, <sup>8</sup> 1996). The bacterial GPAT gene (*plsB*) has also been used to change the fatty acid composition of membrane lipids and to decrease chilling tolerance (Wolter et al, <sup>9</sup> 1992). None of the studies thus far have used GPAT genes to increase oil content in a living organism.

- Because of the unavailability of an ER GPAT gene from plants, we set out to use a plant plastidial GPAT and a bacterial GPAT gene to establish the proof of our concept. Here we demonstrate the use of a plant plastidial or bacterial GPAT gene to increase oil content. Two genes were modified to create four versions to achieve higher oil content.

A cDNA (~gene) for a P-GPAT has been isolated in this lab from safflower (*Carthamus tinctorius*) (Bhella and MacKenzie, <sup>10</sup> 1994). A P-GPAT can use both acyl-ACP and acyl-CoA substrates, although the latter with lesser efficiency (Frentzen, <sup>9</sup> 1993). It was surmised that in the absence of any  
5 acyl-ACP in the cytosol, a P-GPAT will have to use acyl-CoA, although it has to compete with the ER-GPAT for substrates, acyl-CoAs and glycerol-3-phosphate (G-3-P). Its competitive ability could be enhanced by overexpressing it by using a strong constitutive or a tissue specific promoter. Normally, a P-GPAT is targeted to plastids by a transit peptide (TP). The TP  
10 was deleted to confine the GPAT activity in the cytosol (P-GPAT-TP). The modified gene was labelled as *ctpgpat-tp*. It is anticipated that an ER environment may also be required for a P-GPAT-TP to effectively participate in TAG biosynthesis. The ER retention signals (ERRS; Jackson et al., <sup>11</sup> 1990) were added to P-GPAT-TP to meet this requirement. The modified  
15 gene was labelled as *ctpgpat-tp+errs*.

The *Escherichia coli*'s *plsB* gene has been previously characterized (see Wilkison and Bell, <sup>6</sup> 1997). The *plsB* can use both acyl-ACP and acyl-CoA equally effectively. The *plsB* gene was used as such and also with an added  
20 ERRS sequence. The genes were labelled as *plsB* and *plsB+errs*.

#### EXPERIMENTAL

An open reading frame (*orf*) without *tp* (~1.1kb) was PCR amplified from the *ctpgpat* cDNA. Another chimeric gene containing an *errs* at its 3' end was  
25 also PCR amplified. The *orf* of the *E. coli plsB* gene (~2.5kb) was also PCR amplified as it is or with an *errs* at its 3' end. The blunt-end PCR fragments generated using *Pfu* DNA polymerase were cloned into pSK II cloning vector and were sequenced to confirm the incorporation of restriction sites and *errs* sequences to chimeric genes. The *ctpgpat* or *plsB* chimeric genes were  
30 retrieved as *Bam*HI or *Bgl*II segments, respectively, and were cloned into *Bam*HI site of the yeast expression vector, pYES2 (*Invitrogen*), under the

control of a galactose inducible promoter. The INVSc strain (*Invitrogen*) of yeast was transformed with different recombinant constructs.

Yeast cells containing chimeric GPAT genes were grown in a medium containing glucose. GPAT gene expression was induced by transferring cells to growth medium containing galactose. Total proteins from the control and GPAT overexpressing cells were extracted and production of GPAT protein by genes was tested by doing GPAT enzyme assay (Eccleston and Harwood, <sup>12</sup> 1995). The products of the reaction were separated into lyso-phosphatic acid (LPA), phosphatidic acid (PA), di-acyl glycerol (DAG) and TAG by thin layer chromatography. Total lipids in control and cells in which GPAT was overexpressed were measured by <sup>1</sup>H-NMR (Rutar, <sup>13</sup> 1989) and by gas liquid chromatography.

## RESULTS AND DISCUSSION

The modified genes encoded for GPAT activity as shown by an *in vitro* enzyme assay (Table 1). Also, increased GPAT activity led to more lipid production in an *in vitro* assay (Table 2). However, *in vitro* results may not necessarily reflect *in vivo* synthesis as extra substrate is provided in the *in vitro* assay. Nevertheless, we discovered that more lipids were also produced *in vivo* by enhancing the expression of GPAT genes (Tables 3&4).

Here, the proof of the concept was shown in yeast, a eukaryotic model system. Since GPAT is a ubiquitous enzyme for lipid biosynthesis in all living organisms, it is reasonable to expect that the overexpression of these genes will also enhance oil synthesis in other microorganisms and plants. Moreover, in *in vitro* assays, the safflower plastidial GPAT prefers unsaturated fatty acids as compared to *E. coli* GPAT which prefers saturate fatty acids (Table 1). These genes can therefore be used to modify the type of fatty acid at the *sn*-1 position of triglycerides. This will lead to production of structured triglycerides in which the fatty acids occupying each position are limited to only one, or at most, a few specific fatty acids. There is literature evidence

that fatty acid absorption and physiological effect are related to triglyceride structure, not just gross composition.

- Alternatively, the inhibition of GPAT activity by antisense may reduce fats in mammals as higher GPAT activity has been found to be associated with obese rats than with lean rats (Jamadar et al., <sup>14</sup> 1996). Inhibition of GPAT could also be used in plants to reduce lipid content – e.g. reduction of oil in oats would improve shelf life.
- 10 In yeast, especially, oleaginous yeasts, oil content has been increased by choosing a specific carbon and nitrogen source (Ratledge, <sup>15</sup> 1988). The lipid composition has also been changed, for example, in the production of cocoa butter by providing the desired fatty acid in the growth medium as a carbon source (Gierhart, <sup>16</sup> 1984). However, in our process, lipid content has
- 15 been increased just by enhancing the activity of the first enzyme involved in TAG biosynthesis. It appears that more oil is being produced by creation of a sink by higher GPAT activity. Therefore, no manipulation of growth medium, growth conditions or substrates is required to achieve higher lipid content using this process. We also showed that the GPAT has been effective in
- 20 increasing total lipids when two types of acyl-CoA were used. It is likely that these GPATs will also be able to use other acyl-CoAs to increase oil content. This will be especially useful in enhancing GLA (Sinden, <sup>17</sup> 1987) or other specialty lipids in yeast. Alternatively, overexpression of GPATs having a selectivity for specific fatty acids could be used to enhance the content of
- 25 specialty oils in living organisms. The use of lipid enhancing genes such as GPAT may also facilitate the production of commodity oils in microorganisms in an economically sustainable way, a bottleneck thus far (Ratledge, <sup>15</sup> 1988).

#### SUMMARY

- 30 An increase in triglyceride synthesis following expression in yeast of an *E. coli* GPAT and a modified safflower GPAT has been demonstrated by (a) and in

*vitro* assay, (b) NMR analysis of intact yeast and (c) by GC analysis of extracted lipids.

Table 1. Formation of lyso-phosphatidic acid by GPATs

Construct	With 18:1-CoA (pmol/min/mg)		With 16:0-CoA (pmol/min/mg)	
	Glucose	Galactose	Glucose	Galactose
pYES2 (vector)	10.0	6.8	192	131
pYES2:ctpgpat—tp	9.6	33.0	229	175
pYES2:ctpgpat—tp+errs	13.7	44.4	138	157
pYES2: plsB	10.2	28.5	196	790
pYES2: plsB+errs	12.4	20.1	211	346

Table 2. Formation of total lipids by GPATs *in vitro*

Construct	With 18:1-CoA (pmol/min/mg)			With 16:0-CoA (pmol/min/mg)		
	Glucose	Galactose	Ratio*	Glucose	Galactose	Ratio*
pYES2 (vector)	116	112	0.96	1140	513	0.44
pYES2: ctpgpat—tp	184	395	2.15	1170	868	0.74
pYES2: ctpgpat—tp + errs	175	341	1.95	717	771	1.07
pYES2: plsB	98	247	2.51	789	2990	3.77
pYES2: plsB+errs	152	182	1.19	1250	1830	1.46

\*galactose/glucose



Table 3. Estimation of oil in transformed yeasts by NMR

Construct	<sup>1</sup> H-NMR Response*	Ratio of oil contents	% Increase
INVSc (yeast strain)	0.81		
pYES2 (vector)	1.12	1.00	-
pYES2: ctpgpat-tp	1.3	1.17	17
pYES2:ctpgpat-tp+errs	1.68	1.51	51
pYES2: plsB	1.52	1.37	37
pYES2: plsB+errs	1.22	1.10	10

\*Cells grown in galactose/Cells grown in glucose  
NB: values represent ratios relative to pYES2

Table 4. GC Determination of oil content of transformed yeasts (Wt %)

INVSC1		pYES2		ctpgpat-tp		ctpgpat-tp + errs		plsB		plsB+errs	
a	b	a	b	a	b	a	b	a	b	a	b
4.4	4.4	5.0	6.2	5.5	8.8	6.0	7.4	7.5	7.6	5.0	6.2

Data represent the average of two experiments

a and b represent, respectively, transformed cells grown on a glucose medium and cells in which the promoter is induced using a galactose medium. INVSC1 and pYES 2 represent, respectively, wild type yeast and wild type transformed only with the pYES2 plasmid. These constitute two types of control.

NB: Comparisons can be made only between the a and b sets of data because it cannot be guaranteed that the cells are at an identical growth stage in all experiments.

As far as we are aware there are no references in the published literature dealing directly with the modification of oil content and composition by manipulating the expression of a GPAT. However, selected references

5 dealing with increasing seed oil content by the manipulation of other genes are as follows:

1. Zou J., Katavic V., Giblin EM, Barton DL, Mackenzie SL, Keller WA, Hu X., Taylor DC. 1997. Modification of seed oil content and acyl  
10 composition in the Brassicaceae by expression of a yeast *sn*-2 acyltransferase gene. Plant Cell 9:909-923.
2. Zou, J-T, Marillia, E-F, Qi, Q, Barton, DL and Taylor, DC. 1998. Does  
15 mitochondrially-generated acetate contribute to plastidial fatty acid biosynthesis? – Antisense repression of an *Arabidopsis thaliana* mitochondrial pyruvate dehydrogenase kinase (PDHK) gene and its effects on oil content and plant development. B-71. 13<sup>th</sup> International Symposium on Plant Lipids. Seville, Spain, July, 1998.

#### Other References

- 20 3. Frentzen M. 1993. Acyltransferases and Triacylglycerols. In Lipid Metabolism in Plants, Ed TS Moore, Jr. CRC Press, Inc. FL, pp 1195-230.
4. Frentzen M and Wolter FP. 1998. Molecular Biology of Acyltransferases Involved in glycerolipid Synthesis. In Plant Lipid  
25 Biosyntheses: fundamentals and agricultural applications (Ed. JL Harwood). Cambridge University press. pp. 247-272.
5. Wilkison WO and Bell RM 1997. *sn*-Glycerol-3-phosphate acyltransferase from *Escherichia coli*. Biochim Biophys Acta 1348: 3-9.
6. Dircks LK and Sul HS. 1997. Mammalian mitochondrial glycerol-3-  
30 phosphate acyltransferase. Biophys Acta 1348: 17-26.

7. Murata N, Ishizaki-Nishizawa O, Higashi S, Hayashi H, Tasaka Y and Nishida I. 1992. Genetically engineered alteration in the chilling sensitivity of plants. *Nature* 356: 710-713.
8. Nishizawa O. 1996. Chilling-resistant plants and their production. US patent 5,516,667.
9. Wolter FP, Schmidt R and Heinz E. 1992. Chilling sensitivity of *Arabidopsis thaliana* with genetically engineered membrane lipids. *EMBO J* 11: 4685-92.
10. Bhella RS and Mackenzie SL. 1994. Nucleotide sequence of a cDNA from *Carthamus tinctorius* encoding a glycerol-3-phosphate acyl transferase. *Plant Physiol* 106: 1713-1714.
11. Jackson MR, Nilsson T, and Peterson PA. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J* 9: 3153-3162.
12. Eccleston VS and Harwood JL. 1995. Solubilisation, partial purification and properties of acyl-CoA:glycerol-3-phosphate acyltransferase from avocado (*Persea americana*) fruit mesocarp. *Biochimica Biophysica Acta* 1257: 1-10.
13. Rutar V. 1989. Magic angle sample spinning NMR spectroscopy of liquids as a nondestructive method for studies of plant seeds. *J. Agric Food Chem* 37: 70-74.
14. Jamadar SC, Cao WF and Samaniego E. 1996. Relationship between adipose polyamine concentrations and triacylglycerol synthetic enzymes in lean and obese Zucker rats. *Enzyme Protein*:49:222-230.
15. Raffledge C. 1988. Yeasts for lipid production. *Biochem Soc Trans* 16: 1088-1091.
16. Gierhart DL. 1984. Multistage process for the preparation of fats and oils. United States patent 4,485,172.
17. Sinden KW. 1987. The production of lipids by fermentation within the EEC. *Enzyme Microb Technol* 9: 124-125.

## What is Claimed is:

1. A method of increasing the oil content and/or the triglyceride composition in living organisms, comprising over-expressing a gene for the enzyme glycerol-3-phosphate acyltransferase, (GPAT).  
5
2. A method according to Claim 1, wherein the living organism is yeast.
3. A method according to Claim 1, wherein the GPAT gene has the nucleotide sequence of Figure 1.  
10
4. A method according to Claim 1, wherein the gene has the nucleotide sequence of Figure 3.
- 15 5. A method according to Claim 1, wherein the gene has the nucleotide sequence of Figure 5.
6. A method according to Claim 1, wherein the gene has the nucleotide sequence of Figure 7.  
20

GGATCCATGCACGGTCACTCTCGTACATTATCGATGCTCGTTCCGAACAAGATCTTCTTTCTGGAATTCAAAGAGAGTT  
 GGAAGCTGGAACTGCCCCAAACATATTGCTCAAGCAATGGAGGAGCTATATCAGAACTACAAAAATGCAGTTCTCCAAA  
 GTGCGGCTCCTCATGCAGAAGATATTGTGTGTCAACATGCGTGTAGCGTTTGATCGTATGTTCTTGGATGTGAAGGAG  
 CCGTTTGAATTTTACCATATCATGAAGCTATTTTGGAACTTTTAACTACTATATGTTTGGTCAAAATTATATTCGGCC  
 TTTGGTCAATTTCAAGGAATCATACGTTGGCAATGTCTCCGTTTTTCGGTGTAATGGAAGAGCAGCTTAAGCAGGGTGACA  
 AGGTGGTTTTGATCTCAAACCATCAAACAGAAGCAGATCCAGCTGTTATTGCCTTGATGCTTGAAACAACAAACCCCAT  
 ATTTCTGAGAACATAATCTACGTGGCAGGGGATAGAGTAATAACAGATCCTCTTTGCAAGCCTTTAGCATGGGAAGGAA  
 TCTGTTGTGCGTGTATTCAAAAAAGCATATGAATGATGTTCTGAGCTTGCTGAGATGAAAAAAGATCAAATACAAGAA  
 GTTTAAAGGGAGGATGGCTTTGCTTTTGGAGGGCGGATCTAAATAATATGGATTGCGCCAAGTGGTGGCAGGGACAGG  
 CCAGATCCTATCAGAAATCAGTGGTTTCCGGCACCGTTTGATGCCACTTCGCTTGACAACATGAGAAGGCTCGTGGACCA  
 TGCTGGTTTGGTGGGTACATATATCCTTTAGCCATATTGTGCCATGACATCATGCCCTCTCTTCAGGTTGAGAAAG  
 AAATTGGAGAGAAGATTGGATCTCTTTTCATGGCACCGGAATATCAGTGGCACCGGAAATTAATTTCCAAGAAGTTACT  
 GCCTCTTGTGGGTCCCCGAGGAGGCGAAGGCAGCTTATTACAGGCACCTCTATGATTCCGTGTGTGAACAATACAAGGT  
 GCTACATTCTGCGGTACATGGAGGAAAGGGTTAGAAGCATCAACACCAAGTGTCTCGTTGTGACAACCTTGCAGTTTC  
 TCGATTAGGATCC

Figure 1

Nucleotide sequence of the gene *ctpgpat-tp* (*Carthamus tinctorius* plastidial GPAT from which the sequence for a putative transit peptide was removed) used for transforming yeast. Start and stop codon is underlined. ATG was introduced as a start codon in place of GAC.

GSMHGHSTRFIDARSEQDLLSGIQRELEAGTLPRHIAQAMBELYQNYKNAVLSAAPHAEIVLSNMRVAFDRMFLDVKE  
 PFEFSPYHEAILEPFNYMFGQNYIRPLVNFRESYVGNVSVFVGMEEQLKQGDVVLISNRQTEADPAVIALMLETTNPH  
 ISENIYVAGDRVITDPLCKPFSMGRNLLCVYSKKHMNDVPBELAEMKKRSNTRSLKGRMALLRGGSKIWIAPSGGRDR  
 PDPITNQWFPAPFDATSLDNMRRLVDHAGLVGHIYPLAILCHDIMPPLQVEKEIGESWISFHGTGISVAPEINFQEV  
 ASCGSPREAKAAYSQALYDSVCEQYKVLHSAVHGGKGLEASTPSVLSQPLQFLD.D

Figure 2

Translated protein sequence of the gene *ctpgpat-tp* used for transforming yeast. The mature polypeptide will not have the first 90 amino acids of the *ctgpat* (Bhella and MacKenzie, <sup>10</sup> 1994). Amino acid 91, Asp (D), is replaced with Met (M).

GGATCCATGACGGTCACTCTCGTACATTTCATCGATGCTCGTTCCGAACAAGATCTTCTTTCTGGAATTCAAAGAGAGTT  
 GGAAGCTGGAACACTGCCAAAACATATTGCTCAAGCAATGGAGGAGCTATATCAGAACTACAAAATGCAGTTCTCCAAA  
 GTGCGGCTCCTCATGCAGAAGATATTGTGTTGTCAAACATGCGTGTAGCGTTTGATCGTATGTTCTTGGATGTGAAGGAG  
 CCGTTTGAATTTTACCATATCATGAAGCTATTTTGAACCTTTAACTACTATATGTTTGGTCAAAATTATATTCGGCC  
 TTTGGTCAATTTTCAAGGAATCATACGTTGGCAATGTCTCCGTTTTCGGTGTAAATGGAAGAGCAGCTTAAGCAGGGGTGACA  
 AGGTGGTTTTGATCTCAAACCATCAAACAGAAGCAGATCCAGCTGTTATGCTTGATGCTTGAAACAACAACCCCCAT  
 ATTTCTGAGAACATAATCTACGTGGCAGGGGATAGAGTAATAACAGATCCTCTTTGCAAGCCTTTCAGCATGGGAAGGAA  
 TCTGTTGTGCGTGATTTCAAAAAGCATATGAATGATGTTCTGAGCTTGCTGAGATGAAAAAAGATCAAATACAAGAA  
 GTTAAAAGGAGGATGGCTTTGCTTTTGAGGGGCGGATCTAAAATAATATGGATTGCGCAAGTGGTGGCAGGGACAGG  
 CCAGATCCTATCACAAATCAGTGGTTTCCGGCACCCTTTGATGCCACTTCGCTTGACAACATGAGAAGGCTCGTGGACCA  
 TGCTGGTTTTGGTGGGTACATATATCCTTTAGCCATATTGTGCCATGACATCATGCCCCCTCCTCTTCAGGTGAGAAAG  
 AAATTGGAGAGAAGAGTTGGATCTCTTTTCATGGCACCAGGAATATCAGTGGCACCAGGAAATTAATTTCCAAGAAGTTACT  
 GCCTCTTGTGGGTCCCCGAGGAGGCGAAGGCAGCTTATTCACAGGCACCTATGATTCCGTGTGTGAACAATACAAGGT  
 GCTACATCTGCGGTACATGGAGGAAAAGGGTTAGAAGCATCAACACCAAGTGCTCGTTGTGACAAACCAAGCAGAAAC  
 TCGATTAGGATCC

Figure 3

Nucleotide sequence of the gene *ctpgpat-tp+errs* (*Carthamus tinctorius* plastidial GPAT from which the sequence for a putative transit peptide was removed and sequences for endoplasmic reticulum retention signals were added). Start and stop codons are underlined. ATG was introduced as a start codon in place of GAC. Additionally, AAG and AAA (also underlined) in place of TTG and TTT were introduced for ER retention of protein.

GSMHGHSTTFIDARSEQDLLSGIQRELEAGTLPKHIAQAMERLYQNYKNAVLSAAPHAEIVLSNMRVAFDRMFLDVKE  
 PFEFSPYHEAILEPFNYMFGQNYIRPLVNFRESYVGNVSVFGVMEBQLKQGDQVVLISNHQTEADPAVIALMLETNPH  
 ISENIIYVAGDRVITDPLCKPFSMGRNLLCVYSKGMNDVPELAEMKKRSNTRSLKGRMALLLRGGSKIWIAPSGGRDR  
 PDPITNQWFPAPFDATSLDNMRLVDHAGLVGHIYPLAILCHDIMPPLQVEKEIGESWISFRGTGISVAPEINFQEV  
 ASCGSPERAKAAYSQALYDSVCEQYKVLHSAVHGKGLEASTPSVLSQPKQKLD.D

Figure 4

Translated protein sequence of the gene *ctpgpat-tp+errs*. The mature polypeptide will not have the first 90 amino acids of the *ctgp* (Bhella and MacKenzie, 1994). Amino acid 91, Asp (D), is replaced with Met (M). Also Lys (K), underlined, replaced a Phe (F) and Leu (L) at position 3 and 5 from the C terminus.

AGATCTTCCCATGACTTTCTGCTATCCTTGCCGCGCATTGTCATTATTAACCAGAGGCTTTACATCGTTTATGTCCGGCT  
 GGCCACGAATTTACTACAAATTACTGAATTTACCATTAAAGCATCTGGTAAAAAGCAAGTCTATTCCGGCAGATCTGCCC  
 CCGGAACCTGGGGCTGGATACCTCTCGTCCAATTATGTACGTTTACCGTACAACCTCGAAAGCAGATTGTCTGACGTTGCG  
 CGCCAGTGTCTGGCACATGACTTGCTTGACCCGTTAGAGCCGCTGGAAATCGACGCGCAGCTACTGCCGCGCTATGTGT  
 TCATTACGCGCGGCGCGGTGTGTTCACCTATTACACGCGGAAAGAAGTCTATTAAAGCTGTTCCACGACTATCTCGAT  
 TTGCACCGTAGCAACCCAAATCTGGATGTGCAGATGGTGCCAGTGTGCGGTGATGTTTGGTCCGCGCGCGGGGCGTGAAAA  
 AGGCGAAGTGAACCCGCGCTGCGTATGCTTAAACGCGCTACAGAAATTTTTCGCTGTACTGTGGCTCGGTGCGGACAGTT  
 TTGTGCGTTTCTCGCCGTCAAGTTTTCGCTGCGCCGTATGCGCGATGAACACGCGCAGGATAAACTATCGCTCAGAACTG  
 GCGCGCGTGGCGGTATGCACTTTGCCCCGTCAACGCTCTGGCTGCGGTAGGCCACGTTTGCCTGCTCGTCAGGATCTGTT  
 TAATAAGCTGTCTGCGCTCCCGCGCCATTGCCAAAGCGGTAGAAGATGAAGCGCGCAGCAAAAAATCTCCCATGAAAAAG  
 CGCAGCAGAACGCGATTGCACTGTATGGAAGAGATTGCGGCGAATTTCTCTTACGAGATGATTGCGCTGACTGACCGTATT  
 CTGGGCTTCACTGGAACCGACTTTACAGGCGATCAACGCTCCATAACGCTGAGCGCGTTTCGCCAGCTGGCCCCACGACGG  
 TCATGAGCTGGTATATGTGCTTGCACCCGCGAGTCAATGGACTACCTGCTGCTTCTTACGTGCTGTATCACCAGGGGC  
 TGGTCCCGCGCATATCGCCGCGGGATCAACCTGAATTTCTGGCTGCGGGGCGGATTTTCGCCGCTCGGGGGCGTTT  
 TTTATTGCGCGTACGTTTAAAGGCAATAAACTTTATCCACCGTTTTCGGGAGTATCTCGGCGAACTGTTTTCAGCCGTG  
 TTATTCCGTCGAGTACTTCTGGAAGGCGGTGTTTCCCGTACGGGGCGTTTGTGATCCGAAACTGTTACGCTGTGCA  
 TGACCATTACGCGCATGTGCGTGGCGGCGCAGCGTCCGATTACGCTGATTCCGATCTATATCGGTTATGAGCACGTGATG  
 GAAGTGGGTACTTACGCCAAAGAACTGCGCGCGCGACGAAAGAGAAAGAGAGCCTGCGCGAGATGCTGCGCGGTTTAAAG  
 CAAGCTGCGTAATCTCGGTCAAGGTTACGTCAACTTCGGTGAACCAATGCCGTTGATGACCTACCTTAACCAGCATGTAC  
 CTGACTGGCGTGAATCTATCGATCCCATCGAAGCGGTGCGTCCGGCATGTTAACGCCGACGGTCAATAATATTGCTGCC  
 GATCTGATGGTACGCATTAAACAACGCGAGCGCGGCAACGCCATGAACCTGTGCTGTACTGCGCTACTGGCATCAGCTCA  
 GCGCTCACTCACCCGCGAGCAGTTAACCGAGCAACTCACTGCTACCTGGATCTGATGCGCAACGTGCCCTACTCCACGG  
 ACTCTACCGTTTCTTACGCCAGCGCCAGCGAGCTTATCGATCAGCGCGCTGCAATGAACAAGTTTGAAGTCGAGAAAGAC  
 ACAATCGGCGACATCATCTTCTGCGCGCGAGCAAGCGGTGCTGATGACCTACTATCGCAACAACATTGCGCATATGTT  
 GGTGCTGCCCTTCGCTGATGGCGGCAATCGTCACCCAGCATCGCCACATCTCCCGCGACGTTATGATGGAGCAGCTCAATG  
 TGCTTTACCCCAATGCTGAAAGCGGAGCTGTTCTGCGCTGGGATCGCGACGAGTTGCCGACGTTATTGATGCGCTGGCA  
 AATGAGATGCAACGTGAGGGGCTGATTACCTGCAAGATGATGAGTTGCATATCAACCCGCGCATTCTCGCACGCTACA  
 GCTGCTGGCCGCGAGCGCGCGGAAACGCTGCAACGTTATGCCATCACCTTCTGGTTGTTGAGTGCCAACCCGCTCGATCA  
 ACCGCGGTACGCTGGAGAAAGAGAGCGCGCACGCTGCGCAACGCTCTCCGTGCTGCACGGCATCAACGCGCGGAGTTC  
 TTCGACAAGCGGTGTTTCACTTCTGCTGCTGACACTGCGTGATGAAGGTTATCAGCGATAGCGGCGATGCCGAACC  
 GGCAGAAACGATGAAGGTTTATCAGTTGCTGGCGGAGTTGATTACATCAGACGTGCGTTTGACGATTGAGAGTGCAGCG  
 AGGGCGAAGGGTATTCAGATCT

Figure 5

Nucleotide sequence of the gene *plsB* (*E. coli* GPAT). Start and stop codon is underlined.

00132762:002150

MTFCYPCRAFALLTRGFTSEMSGWPRIYYKLLNPLSILVKSISIPADPAPELGLDTSRPIMYVLPYNSKADLLTLRAQC  
LAHDLDPDLEPLEIDGTLLPRYVFIHGGPRVFTYYTPKEESIKLPHDYLDLHRSNPNLDVQMVPVSVMFGRAPGREKGEV  
NPPLRMLNGVQKFFAVLNLGRDSFVRFPSVSLRRMADEHGTDKTIQKLAARVARMHFARQRLAAVGPRLPARQDLFNKL  
LASRAIAKAVEDEARSKKISHEKAQONAIALMEEIAANFSYEMIRLTDRILGFTWNRLYQGINVHNAERVRLAHGHEL  
VYVPCHRSHMDYLLLSYVLYHQGLVPPHIAAGINLFWPAGPIFRRLGAPFIRRTFKGNKLYSTVFREYLGELFSPRGYSV  
EYFVEGGRSRTGRLLDPKTGTLSTIQAMLRGGTRPITLPIYIGYEHVMEVGTAKELRGATKEKESLPQMLRGLSKLR  
NLGQGYVNFGEPMPLMTYLNQHVDPWRESIDPIEAVRPWLTPVTNNIAADLMVRINNAGAAANAMNLCCTALLASRQSL  
TREQLTEQLNCYLDLMRNVPYSTDSTVPSASASELIDHALQMNKFEVEKDTIGDIIILPREQAVLMTYYRNNAHMLVLP  
SLMAAIVTQHRHISRDVLMHVNVLYPMLKAELFLRWDRDELDPVIDALANENQRQGLITLQDDELHINPAHSRTLQLLA  
AGARETLQRYAITFWLLSANPSINRGTLKESRTVAQRLSVLHGINAPEFFDKAVFSSLVLTLRDEGYISDSGDARPAET  
MKVYQLLAELITSDVRLTIESATQEG.SD

Figure 6

Translated protein sequence of the gene *plsB* (*E. coli* GPAT).

00133768-002499



AGATCTTCCCATGACTTTCTGCTATCCTTGCCGCGCATTTGCATTATTAACCAAGAGGCTTTACATCGTTTATGTCCGGCT  
 GGCCACGAATTTACTACAAATTACTGAATTTACCATTAAAGCATCCTGGTAAAAAGCAAGTCTATTCCGGCAGATCCTGCC  
 CCGGAACCTGGGGCTGGATACCTCTCGTCCAATTATGTACGTTTACCCTGACAACTCGAAAGCAGATTTGCTGACGTTGCG  
 CGCCAGTGCTGTCACATGACTTGCCTGACCCGTTAGAGCCGCTGGAATCGACGGCAGCTACTGCCGCGCTATGTGT  
 TCATTACGGCGGCGCGCTGTGTTACCTATTACACGCGGAAAGAGAGTCTATTAAGCTGTTCCACGACTATCTCGAT  
 TTGCACCGTAGCAACCCAAATCTGGATGTGCAGATGGTGCCAGTGTGGTGTATTTGGTCCGGCGCGGGCGGTGAAAA  
 AGGCGAAGTGAACCCGCGCTGCGTATGCTTAAACGGCGTACAGAAATTTTTCGCTGACTGTGGCTCGGTGCGACAGTT  
 TTGTGCGTTTCTCGCGTCACTTTCGCTGCGCGTATGGCGGATGAACACGGCAGGATAAACTATCGCTCAGAACTG  
 GCGCGCTGGCGCGTATGCACTTTGCCCGTCAACGTCGCTGCGTAGGCCACGTTTGCTGCTCGTCAGGATCTGTT  
 TAATAAGCTGCTGCGCTCCCGCGCCATTGCCAAAGCGGTAGAAGTGAAGCGCGCAGCAAAAAAATCTCCCATGAAAAAG  
 CGCAGCAGAACCGGATTGCTGCTGATGGAAGAGATTGCGGCGAATTTCTCTTACGAGATGATTGCGCTCGGTGCGACAGTT  
 CTGGGCTTCACTGGAACCGACTTTACCAGGGCATCAACGTCCTAAGCTGAGCGGTTCCGCGAGCTGGCCACGACGG  
 TCATGAGCTGGTATATGTGCTTGGCACCGCAGTCACATGGAATACTGCTGCTTCTTACGCTGTATCACCAGGGGC  
 TGGTGGCGCGCATATCGCCGCGGGATCAACCTGAATTTCTGGCTGCGCGCGGATTTCGCGCGTCTGGGGCGGTTT  
 TTTATTCGCGTACGTTTAAAGGCAATAAATCTTATTCACCGTTTTCGCGGAGTATCTCGCGCAACTGTTGAGCGGTGG  
 TTATTCGCTCGAGTACTTCTGGAAGGCGGTGTTCCCGTACGGGCGGTTTGTGGATCCGAAAACTGGTACGCTGTGCA  
 TGACCATTCAGGCGATGCTGCGTGGCGGCGACGCGTCCGATTACGCTGATTCCGATCTATATCGGTTATGAGCAGCTCATG  
 GAAGTGGGTACTTACGCCAAAGAACTGCGCGGCGGACGAAAGAGAGAGCCTGCCGAGATGCTGCGCGGTTTAAAG  
 CAAGCTGCGTAATCTCGGTACGGTTACGTCAACTTCGGTGAACCAATGCGCTGATGACCTACCTTAACGACGCTATC  
 CTGACTGGCGTGAATCTATCGATCCCATCGAAGCGGTGCGTCCGCGATGGTTAACGCCGACGGTCAATAATATTGCTGCC  
 GATCTGATGGTACGCATTAAACGCGAGCGCGGCAACGCCATGAACCTGTGCTGTACTGCGCTACTGGCATCAGTCA  
 GCGCTCACTCACCCGCGAGCAGTTAACCGAGCAACTCAACTGCTACCTGGATCTGATGCGCAACGTTGCCCTACTCCACGG  
 ACTCTACCGTTCTTTCAGCCAGCGCCAGCGAGCTTATCGATCACGCGCTGCAAATGAACAAGTTTGAAGTCGAGAAAGAC  
 ACAATCGGCGACATCATCTTCTGCGCGCGAGCAAGCGGTGCTGATGACCTACTATCGCAACAACATTGCGCATATGTT  
 GGTGCTGCCCTTCTGCTGATGGCGGCAATCGTCACCCAGCATCGCCACATCTCCCGCGACGTATTGATGGAGCAGTCAATG  
 TGCTTTACCAATGCTGAAAGCGGAGCTGTTCTGCGCTGGGATCGCGACGAGTTGCCGGACGTTATTGATGCGCTGGCA  
 AATGAGATGCAACGTCAGGGGCTGATTACCTTGCAAGATGATGAGTTGCATATCAACCCGGCGCATTTCTGCGACGCTACA  
 GCTGCTGGCGCGAGCGCGCGGAAACGCTGCAACGTTATGCCATCACCTTCTGTTGTTGAGTGCCAACCCGTCGATCA  
 ACCCGGTTACGCTGGAGAAAGAGAGCCGACCGTCGCGCAACGTCCTCTCGTGTGACGGCATCAACGCGCCGAGTTT  
 TTCGACAAGGCGGTGTTCACTTCTCTGCTGCTGACACTGCGTGATGAAGGGTATATCAGCGATAGCGCGGATGCCGAACC  
 GGCAGAAACGATGAAGGTTTATCAGTTGCTGGCGGAGTTGATTACATCAGACGTGCGTTTGACGATTGAGAGTGCGAAGC  
 AGAAGGAAGGTAATCAGATCT

Figure 7

Nucleotide sequence of the gene *plsB+errs* (*E. coli* GPAT to which sequences for  
 endoplasmic reticulum retention signals were added). Start and stop codons are  
 underlined. AAG and AAG (also underlined) in place of GGC and ACG were  
 introduced at the 3' end for ER retention of protein.

MTFCYPCRAFALLTRGFTSFMSGWPRIYYKLLNLPLSILVKSISIPADPAPELGLDTSRPIMYVLPYNSKADLLTLRAQC  
LAHDLPDPLEPLEIDGTLLPRYVFIHGGPRVFTYYTPKEESIKLFHDYLDLHRSNPNLDVQMVPVSVMFGRAPGREKGEV  
NPPLRMLNGVQKFFAVLWLGSDSFVRFPSPSVSLRRMADEHGTDKTIAQKLARVARMHFARQRLAAVGPRLPARQDLFNKL  
LASRAIAKAVEDEARSKKISHEKAQQNAIALMEEIAANFSYEMIRLTDRILGFTWNRLYQGINVHNAERVRLAHDGHEL  
VYVPCRSHMDYLLLSYVLYHQGLVPPHIAAGINLNFWPAGPIFRRLGAFFIRRTFKGNKLYSTVFREYLGELFSRGYSV  
EYFVEGGRSRTGRLLDPKTGTLSTMTIQAMLRGGTRPITLIPIYIGYEHVMEVGTYAKELRGATKEKESLPQMLRGLSKLR  
NLGQGYVNFGEPMPLMTYLNQHVDPDWRESIDPIEAVRPWLTPTVNNIAADLMVRINNAGAANAMNLCCTALLASRQSL  
TREQLTEQLNCYLDLMRNVPISTSTVPSASASELIDHALQMNKFEVEKDTIGDIIILPREQAVLMTYRNNTIAHMLVLP  
SLMAAIVTQHRHISRDVLMHVNVLYPMLKAEFLRWDRDELDPVIDALANEMQRQGLITLQDDELHINPAHSRTLQLLA  
AGARETLQRYAITFWLLSANPSINRGTLKESRTVAQRLSVLHGINAPEFFDKAVFSSLVLTLRDEGYISDSGDAEPAET  
MKVYQLLAELITSDVRLTIESAKOKEG.SD

### Figure 8

Translated protein sequence of the gene *plsB+errs*. Amino acid Lys (K),  
underlined, replaced a Gly (G) and Thr (T) at position 3 and 5 from the C  
terminus.